Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Virological Methods 124 (2005) 73–77
Journal of Virological Methods
www.elsevier.com/locate/jviromet

The use of reverse transcriptase for efficient first- and second-strand cDNA synthesis from single- and double-stranded RNA templates

I.E. Tzanetakis[a], K.E. Keller[b], R.R. Martin[a,b,*]

[a] *Department of Botany and Plant Pathology and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331, USA*
[b] *USDA-ARS-HCRL, Corvallis, OR 97330, USA*

Received 21 July 2004; received in revised form 26 October 2004; accepted 9 November 2004
Available online 15 December 2004

Abstract

Molecular characterization of eight distinct, difficult-to-clone RNA plant viruses was accomplished after the development of a reverse transcriptase-based first- and second-strand cDNA synthesis method. Double-stranded (ds) RNA templates isolated from strawberry and blackberry and several herbaceous hosts (mint, pea and tobacco) were cloned using this method. Templates, combined with random primers, were denatured with methyl mercuric hydroxide. Reverse transcriptase was added followed by the addition of RNase H. The resulting dsDNA was then digested with restriction endonucleases to produce shorter fragments that could be cloned efficiently into a T-tailed vector after adding an A-overhang using *Taq* polymerase. This procedure resulted in a high number of cloned fragments and allowed insert sizes up to three kilobase-pairs. Unlike traditional cDNA construction methods, there is no need for additional enzymes/steps for second-strand synthesis, PCR amplification or prior sequence information. Synthesis and cloning of cDNA derived from dsRNA templates is much more efficient than with previously described methods. This procedure also worked well for cloning gel-purified dsRNA and with single-stranded RNA templates. Published by Elsevier B.V.

Keywords: dsRNA; RNA virus cloning; cDNA synthesis; Reverse transcriptase

1. Introduction

Molecular characterization of plant viruses, the majority of which have a RNA genome, developed rapidly after the discovery that reverse transcriptase (RT) catalyzed the formation of DNA from an RNA template (Baltimore, 1970; Temin and Mizutani, 1970). The characterization of RNA viruses that are recalcitrant to purification relies largely on the ability to purify virus-specific double-stranded (ds) RNAs from a wide variety of plant hosts (Morris and Dodds, 1979; Bar-Joseph et al., 1983; Choi and Randles, 1997; Valverde et al., 1990). dsRNA molecules are formed during virus replication, and are comprised of full-length copies of the genomic and subgenomic RNAs of sense and ambisense RNA viruses. However, the quality of dsRNA templates is influenced greatly by the host plant from which they are extracted. Many plants contain compounds such as glycosides, polyphenols, and polysaccharides that co-purify with nucleic acids and are inhibitory towards enzymatic reactions. Many purification techniques have been developed to address the problems caused by inhibitors (Agudo et al., 1995; Baker et al., 1990; Do and Adams, 1991; Koonjul et al., 1999; Rowland and Nguyen, 1993; Scott and Playford, 1996; Tesniere and Vayda, 1991; Thompson et al., 2003), but are not always successful to eliminate these compounds.

The widest use of reverse transcriptase in cloning has been the exploitation of the RNA-dependent DNA polymerase activity to catalyze first-strand cDNA synthesis from RNA templates. Typical protocols for the generation of first- and second-strand cDNA call for the use of reverse transcriptase for first-strand synthesis followed by treatment with RNase H before second-strand synthesis utilizing *E. coli* DNA polymerase I (Jelkmann et al., 1989). Various methods have been

* Corresponding author. Tel.: +1 541 738 4041; fax: +1 541 738 4025.
E-mail address: martinrr@science.oregonstate.edu (R.R. Martin).

0166-0934/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jviromet.2004.11.006

described for manipulation of the double-stranded cDNA following second-strand synthesis, including adapter/linker ligation, homopolymer tailing, and generation of blunt ends for cloning into appropriate vectors (Gubler and Hoffman, 1983; Jelkmann et al., 1989; Okayama and Berg, 1982). Ligation of an adapter to dsRNA in order to provide a known priming site has also been described (Lambden et al., 1992; Potgieter et al., 2002; Vreede et al., 1998). Some cDNA synthesis/cloning protocols use PCR amplification to obtain sufficient template concentration to allow efficient cloning into plasmid vectors (Koonjul et al., 1999; Ono and Nakane, 1990). There are several disadvantages to these methods, including the number of steps involved and the presence of inhibitory compounds that co-purify with nucleic acids that can hinder cDNA synthesis and/or PCR amplification (Tesniere and Vayda, 1991). Additionally, gel purification of dsRNA bands can introduce inhibitors of second-strand cDNA synthesis (Tzanetakis, personal observation; Potgieter et al., 2002), while first- and second-strand synthesis using reverse transcriptase appears to be unaffected (this publication).

Reverse transcriptase is both an RNA- and DNA-dependent DNA polymerase. A method for cDNA synthesis was developed that utilizes both the RNA- and DNA-dependent activities of RT. This method circumvents many steps typically involved in cDNA synthesis from dsRNA templates and avoids problems with inhibitors often encountered when using other DNA polymerases, such as DNA polymerase I or *Taq* polymerase. This method also generates a high percentage of clones containing large inserts.

2. Materials and methods

2.1. Virus isolates

As a test of the procedure described below, we purified dsRNA of eight viruses from the following host plants: (1) An unknown virus of pea obtained from Dr. Richard Hampton which caused a calico-type mosaic and was maintained by mechanical transmission in *Pisum sativum* 'Early Freezer 680' (EF680), referred to as pea calico. (2) Fragaria chiloensis latent virus (FClLV), obtained from the USDA-ARS National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, accession number CFRA 9089 (Germplasm Resources Information Network (GRIN) database), maintained in *Fragaria chiloensis*. (3–6) Four viruses cloned from dsRNA purified from clones of *Mentha* sp. (MEN 454.001 and MEN 454.004, GRIN database) obtained from the USDA-ARS NCGR in Corvallis, Oregon, showing symptoms typical of virus infection. One of these four, identified as Strawberry latent ringspot virus (SLRSV) (Postman et al., 2004), was transferred mechanically to *Nicotiana occidentalis* for virus purification. (7 and 8) Beet pseudo-yellows virus (BPYV) and Blackberry yellow vein associated virus (BYVaV) were cloned from dsRNA isolated from blackberry, *Rubus* sp., 'Apache' and 'Chickasaw', obtained from commercial fields in South Carolina and Arkansas, respectively, that showed leaf chlorosis and vein-bending symptoms.

2.2. ss- and dsRNA isolation

For single-stranded RNA, SLRSV was purified from *N. occidentalis* plants according to the method of MacDonald et al. (1991) and the RNA was extracted from purified virions utilizing the RNeasy kit (Qiagen, Valencia, CA). Two methods were used for isolation of dsRNA. Isolation from pea was performed using the methods of Choi and Randles (1997) and Yoshikawa and Converse (1990) that are based on the binding of nucleic acids to cellulose in the presence of ethanol. dsRNA from all other hosts was extracted according to the latter method only.

2.3. cDNA synthesis

dsRNA was utilized as templates for cDNA with or without gel purification. Gel separation was performed in Tris phosphate–EDTA (TPE) agarose gels. After ethidium bromide staining, dsRNA bands were excised and gel-purified using GeneClean Spin Kit #1101-400 (Qbiogene, Inc., Carlsbad, CA). The water-eluted dsRNA was reduced to a dried pellet in vacuo (Savant Integrated Speed Vac System ISS110, Thermo Forma, Marietta, OH). Dried ds- and ssRNA pellets were resuspended in a denaturant mix containing 20 mM CH_3HgOH and one microgram random hexamer primers in a total volume of seven microlitres. This mixture was incubated for 30 min at room temperature. Forty-three microlitres of previously assembled reverse transcriptase solution containing 50 mM Tris acetate (pH 8.4), 75 mM potassium acetate, 8 mM magnesium acetate, 20 mM DTT, 0.4 mM dNTPs, and 200 U Superscript III RT (Invitrogen, Carlsbad, CA) was added to the denaturant mix. The solution was vortexed, centrifuged briefly, left at room temperature for 2 min and then incubated at 50 °C for 60 min. Following the incubation period, 4 U RNase H were added to the reaction that was then placed at 37 °C for 90 min.

To obtain shorter DNA fragments which would clone more efficiently, a mixture of *Aat*II, *Eco*NI, *Hpa*I and *Nco*I restriction endonucleases (20 U each) were also added to the reaction. Because of buffer incompatibility in subsequent steps, the Rapid PCR Purification System (Marligen, Ijamsville, MD) was used to purify the DNA fragments. Due to the nature of the vector used for ligation (TA vector with a thymidine overhang to facilitate efficient cloning), a deoxyadenosine overhang was added to the cDNA utilizing *Taq* polymerase (Xhang and Rowhani, 2000). The reaction consisted of 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 2.5 U *Taq* DNA polymerase (New England Biolabs, Beverly, MA), and 0.02 mM each of the dNTPs, used in order to fill in the single-stranded DNA overhangs formed by digestion with restriction endonucleases and add the deoxyadenosine overhang. The reaction was incubated at 75 °C for 30 min, followed

by removal of enzyme and nucleotides with the Rapid PCR Purification System (Marligen, Ijamsville, MD). Total volume of the eluted products was reduced to a final volume of 4–20 μl in vacuo.

2.4. Ligation and transformation

All cDNA ligations and transformations were done using Vector TOPO pCR 4.0 cloning kits (Invitrogen, Carlsbad, CA). Four microlitres of the final cDNA synthesis product was used in the recommended ligation reaction. This mixture was incubated for 30 min at 12 °C, followed by 30 min at room temperature. Two microlitres of the ligation solution was used to transform chemically competent cells. DNA was isolated (Perfectprep Plasmid Mini #0032 005. 500, Brinkman, Westbury, NY) from 2-ml overnight cultures containing 25 μg/ml kanamycin.

2.5. Sequencing and analysis

DNA sequencing was performed by Macrogen Inc. (Seoul, Korea) in an ABI 3730 XL automatic DNA sequencer. Sequence identification and genome assembly were completed utilizing the BLAST (Altschul et al., 1997) and CAP3 (Huang, 1996) programs.

3. Results

Partial sequence was obtained for eight viruses using the procedure described above. The sequence information allowed for a tentative assignment of the viruses to genera providing insight into possible genome organization and contig arrangement. With this information, primers were designed for RT-PCR to fill in gaps and for 5′ and 3′ RACE to obtain end sequence information, thus obtaining entire sequences of four of these viruses. Complete sequences of FClLV (family *Bromoviridae*, genus *Ilarvirus*), Mint virus X (family *Flexiviridae*, genus *Potexvirus*), Mint virus 1 (family *Closteroviridae*, genus *Closterovirus*) and BYVaV (family *Closteroviridae*, genus *Crinivirus*) were obtained using the sequence information acquired with this method. About two kilobases of sequence was obtained by sequencing 20 or fewer cDNA clones for each of the other unknown viruses, Mint vein-bending virus (family *Closteroviridae*, unassigned genus), pea calico (genus *Umbravirus*) and Strawberry latent ringspot virus (tentative member of the family *Comoviridae* (ICTV) but with sequence homology with *Sequiviridae*). Less sequence data was obtained from Beet pseudo-yellows virus where only five cDNA clones were sequenced (Table 1).

Endonucleases chosen for cloning experiments have optimal activity in buffer systems similar to that of the reverse transcription reaction. Any restriction endonuclease compatible with this buffer system may also be used. The cloned fragment sizes were affected by use of these enzymes. Elimination of restriction endonucleases or use of fewer than four resulted in larger-sized cloned fragments. Exclusion of restriction enzymes allowed cloning of fragments of up to three kilobases. The average size of the fragments cloned, utilizing all four endonucleases, was approximately 600 bp compared to a 1300 bp average in their absence. Without endonuclease treatment, fewer fragments were cloned due to less efficient topoisomerase-mediated ligation of the larger fragments.

4. Discussion

The ability to clone cDNA, derived from pea calico dsRNA templates purified from pea, demonstrated the advantages of this method, given the inability to obtain any sequence data following multiple cloning attempts utilizing many of the previously described procedures (Zhang and Rowhani, 2000; Davis and Boyle, 1990; Jelkmann et al., 1989; Qian and Kibenge, 1994).

Utilizing the protocol presented here, sequence information from eight RNA viruses infecting five different plant species was obtained. Small fruits crops such as strawberry and blackberry are known to contain inhibitors of in vitro enzymatic reactions that co-purify with total RNA and dsRNA, regardless of isolation method. Gel purification of dsRNA may also lead to inhibition of downstream reactions, thus reducing cloning efficiency (Potgieter et al., 2002).

When carrying out RT-PCR reactions with total RNA isolated from *Fragaria*, *Rubus* and *Vaccinium* sp., it was ob-

Table 1
Sequence information for eight viruses obtained using the dsRNA cloning procedure described here

Virus[a]	Host	No. of clones sequenced/average size	Sequence ID number in GenBank
BPYV	*Rubus* sp. 'Apache'	5/800	–[b]
BYVaV	*Rubus* sp. 'Chickasaw'	43/650	AY682104
FClLV	*Fragaria chiloensis*	15/600	AY682102
MV-1	*Mentha* sp.	20/550	AY789139
MVBV	*Mentha* × *gracilis*	25/800	AY548137
MVX	*Mentha* × *gracilis*	15/500	AY682101
Pea calico	*Pisum sativum*	60/600	AY686696
SLRSV	*Mentha* × *gracilis*	50/1300	AY682103

[a] Virus acronyms: BPYV = Beet pseudo-yellows virus; BYVaV = Blackberry yellow vein associated virus; FClLV = Fragaria chiloensis latent virus; MV-1 = Mint virus 1; MVBV = Mint vein-bending virus; MVX = Mint virus X; SLRSV = Strawberry latent ringspot virus.
[b] The sequence of this clone is >99% identical to BPYV GenBank ID NC 005210.

served that inhibition occurred at the PCR rather than the RT step. Further dilution of the RT reactions often resulted in a positive RT-PCR result or a much more intense band when the PCR product was analyzed. Also, it was observed that the inhibited step in the cDNA synthesis procedure was the second-strand synthesis, as PCR amplification was often successful when utilizing cDNA derived from dsRNA templates from *Fragaria* and *Rubus* sp. These observations suggested that reverse transcriptase is perhaps more tolerant of inhibitory compounds than other DNA polymerases and that the latter may be inhibited during the production of dsDNA from dsRNA templates. M-MLV reverse transcriptase will synthesize a complementary DNA strand from single-stranded RNA or DNA (Verma, 1975). These factors led to the hypothesis that DNA polymerase I may be inhibited in traditional cloning strategies, leading us to attempt second-strand synthesis using RT.

To assess the efficiency of the new procedure with ssRNA templates, Strawberry latent ringspot virus single-stranded RNA from purified virions was utilized. The procedure yielded cloning efficiencies higher than that of dsRNA templates of the same or other viruses. While the amount of template used was comparable to that of dsRNA templates, elimination of enzymatic inhibitors during virus purification and unproblematic denaturation of the ssRNA secondary structure versus that of dsRNA apparently led to unrestricted enzymatic activity and high cloning efficiency.

This simple, yet efficient method has allowed cloning of eight previously unidentified viruses or viruses where nucleic acid sequence data was lacking. Elimination of multiple, complex enzymatic reactions and the ability to perform double-stranded cDNA synthesis utilizing only a few common laboratory enzymes makes this method simple and time/cost efficient, while at the same time eliminating problems that can be encountered when working with dsRNA templates.

Acknowledgements

We would like to thank Drs. Robert Doss, Joyce Loper and Jennifer Kraus for reviewing and providing helpful suggestions on the manuscript.

References

Agudo, L.C., Gavidia, I., Pérez-Bermúdez, P., Segura, J., 1995. PEG precipitation, a required step for PCR amplification of DNA from wild plants of Digitalis obscura L. BioTechniques 18, 766–768.

Baker, S.S., Rugh, C.L., Kamalay, J.C., 1990. RNA and DNA isolation from recalcitrant plant tissues. BioTechniques 9, 268–272.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, J.D., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Baltimore, D., 1970. Viral RNA-dependent DNA polymerase. Nature 226, 1209–1211.

Bar-Joseph, M., Rosner, A., Moscovitz, M., Hull, R., 1983. A simple procedure for the extraction of double-stranded RNA from virus-infected plants. J. Virol. Methods 6, 1–8.

Choi, Y.G., Randles, J.W., 1997. Microgranular cellulose improves dsRNA recovery from plant nucleic acid extracts. BioTechniques 23, 610–611.

Davis, V.S., Boyle, J.A., 1990. Adapting the polymerase chain reaction to a double-stranded RNA genome. Anal. Biochem. 189, 30–34.

Do, N., Adams, R.P., 1991. A simple technique for removing plant polysaccharide contaminants from plant DNA. BioTechniques 10, 162–166.

Gubler, U., Hoffman, B.J., 1983. A simple and very efficient method for generating cDNA libraries. Gene 25, 263–639.

Huang, X., 1996. An improved sequence assembly program. Genomics 33, 21–31.

Jelkmann, W., Martin, R.R., Maiss, E., 1989. Cloning of four plant viruses from small quantities of double-stranded RNA. Phytopathology 79, 1250–1253.

Koonjul, P.K., Brandt, W.F., Farrant, J.M., Lindsey, G.G., 1999. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. Nucleic Acids Res. 27, 915–916.

Lambden, P.R., Cooke, S.J., Caul, E.O., Clarke, I.N., 1992. Cloning of noncultivatable human rotavirus by single primer amplification. J. Virol. 66, 1817–1822.

MacDonald, S.G., Martin, R.R., Bristow, P.R., 1991. Characterization of an ilarvirus associated with a necrotic shock reaction in blueberry. Phytopathology 81, 210–214.

Morris, T.J., Dodds, J.A., 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69, 854–858.

Okayama, H., Berg, P., 1982. High efficiency cloning of full length cDNA. Mol. Cell. Biol. 2, 161–170.

Ono, K., Nakane, H., 1990. Mechanisms of inhibition of various cellular DNA and RNA polymerases by several flavonoids. J. Biochem. 108, 609–613.

Postman, J.D., Tzanetakis, I.E., Martin, R.R., 2004. First report of Strawberry latent ringspot virus in Mentha from North America. Plant Dis. 88, 907.

Potgieter, A.C., Steele, A.D., van Dijk, A.A., 2002. Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes. J. Gen. Virol. 83, 2215–2223.

Qian, B., Kibenge, F.S., 1994. Observations on polymerase chain reaction amplification of infectious bursal disease virus dsRNA. J. Virol. Methods 47, 237–242.

Rowland, L.J., Nguyen, B., 1993. Use of polyethylene glycol for purification of DNA from leaf tissue of woody plants. BioTechniques 14, 735–736.

Scott, K.D., Playford, J., 1996. DNA extraction technique for PCR in rain forest plant species. BioTechniques 20, 974–979.

Temin, H.M., Mizutani, S., 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226, 1211–1213.

Tesniere, C., Vayda, M.E., 1991. Method for the isolation of high-quality RNA from grape berry tissues without contaminating tannins or carbohydrates. Plant Mol. Biol. Rep. 9, 242–251.

Thompson, J.R., Wetzel, S., Klerks, M.M., Vaskova, D., Schoen, C.D., Spak, J., Jelkmann, W., 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in Fragaria spp. in combination with a plant mRNA specific internal control. J. Virol. Methods 111, 85–93.

Valverde, R.A., Nameth, S.T., Jordan, R.L., 1990. Analysis of double stranded RNA for plant virus diagnosis. Plant Dis. 74, 255–258.

Verma, I.M., 1975. Studies on reverse transcriptase of RNA tumor viruses III properties of purified moloney murine leukemial virus DNA polymerase and associated RNase H. J. Virol. 15, 843–854.

Vreede, F.T., Cloete, M., Napier, G.B., van Dijk, A.A., Viljoen, G.J., 1998. Sequence-independent amplification and cloning of large dsRNA virus genome segments by poly(dA)-oligonucleotide ligation. J. Virol. Methods 72, 243–247.

Yoshikawa, N., Converse, R.H., 1990. Strawberry pallidosis disease: distinctive dsRNA species associated with latent infections in indicators and in diseased strawberry cultivars. Phytopathology 80, 543–548.

Zhang, Y.P., Rowhani, A., 2000. A strategy for rapid cDNA cloning from double-stranded RNA templates isolated from plants infected with RNA viruses by using Taq DNA polymerase. J. Virol. Methods 84, 59–63.